PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 47/18, 47/26, 38/37, A61L 2/04

A1

(11) International Publication Number: **WO 99/10011**

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/AU98/00682

(22) International Filing Date: 25 August 1998 (25.08.98)

(30) Priority Data:
PO 8719 25 August 1997 (25.08.97) AU

(71) Applicant *(for all designated States except US)*: CSL LIMITED [AU/AU]; 45 Poplar Road, Parkville, VIC 3052 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KANELLOS, Jerry [AU/AU]; 27 Myrtle Street, Clifton Hill, VIC 3068 (AU). OATES, Adrian [AU/AU]; 11 Ann Street, Prahran, VIC 3181 (AU). GOSS, Neil [AU/AU]; 39 Glenard Drive, Eaglemont, VIC 3084 (AU).

(74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: DRIED BIOLOGICALLY OR THERAPEUTICALLY ACTIVE PREPARATIONS

(57) Abstract

A dried, heat–treated product comprises (i) a heat labile, biologically or therapeutically active protein or peptide preparation and (ii) a stabilising effective amount of a composition comprising sucrose, trehalose and at least one amino acid. The protein or peptide preparation may be, for example, a Factor VIII concentrate or a von Willebrand Factor concentrate.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

DRIED BIOLOGICALLY OR THERAPEUTICALLY ACTIVE PREPARATIONS

FIELD OF THE INVENTION

This invention relates to dried biologically or therapeutically active preparations, and in particular it relates to dried or lyophilised Factor VIII preparations. The present invention provides in particular for the stabilisation of such Factor VIII preparations which are to be subjected to a terminal dry heat treatment step, such as heating at 80°C for 72 hrs, used as a viral inactivation step.

BACKGROUND OF THE INVENTION

Classic haemophilia, haemophilia A, is an X chromosome-linked disorder of blood coagulation which causes a decrease in functional levels of a glycoprotein known as antihaemophilic factor (AHF) or Factor VIII (FVIII) (Levine 1987).

FVIII is a cofactor for Factor IXa in the activation of Factor X and is crucial for activation of the intrinsic coagulation pathway (Foster and Zimmerman, 1989). FVIII is present in the plasma of normal individuals at a level of 1 unit/mL (100 ng/mL). It circulates in plasma in an inactive form bound to another high molecular weight glycoprotein, the FVIII-related antigen, von Willebrand Factor (vWF) at a molar ratio of approximately 1:100. vWF is deficient or abnormal in patients with von Willebrand's disease. Mature vWF consists of 2050 amino acids residues and circulates in plasma as a series of disulfide-linked multimers between 1 to $20x10^6$ daltons. The association with vWF serves to stabilise the FVIII molecule in plasma as well as localise the clotting cascade to the site of vascular damage through vWF adhesion to the activated platelet surface (Weiss 1977).

The molecular weight of FVIII in plasma ranges between 250,000 to 300,000 daltons. It is composed of heavy and light chains with molecular weights of approximately 90 and 80 kDa respectively, separated by a heavily glycosylated β-domain region of approximately 130 kDa (Fulcher and Zimmerman 1982, Rotblat *et al.* 1985). FVIII is susceptible to proteolytic cleavage by thrombin and Factor Xa. This serves to remove the β-domain, resulting in a conformational change whereby the heavy and light chains become linked by calcium ion binding. This cleavage correlates with an increase in functional activity and represents the activated form of the molecule, FVIIIa (Eaton *et al.* 1986, Fulcher *et al.* 1985, Andersson *et al.* 1986, Fay *et al.* 1986, Pittman and Kaufman 1986).

Once FVIIIa has fulfilled its roles, it is proteolytically cleaved further by either thrombin, FACTOR Xa or activated protein C, correlating with loss of functional activity. Peptides range from 43 kDa to 80 kDa form, depending on the participating enzyme (Eaton *et al.* 1987).

The cloning of human FVIII gene and the expression of active recombinant FVIII have been reported. The cDNA codes for a single chain FVIII consist of 2332 amino acid residues with a molecular weight of 264,763 Da prior to glycosylation and the glycosylated form of approximately 330 kDa (Toole *et al.* 1984, Gitschier *et al.* 1984, Rotblat *et al.* 1985). It has been shown that calcium is necessary for maintaining the association of FVIII heavy and light chains, and the removal of calcium from FVIII preparations has been demonstrated to result in loss of procoagulant activity (Fass *et al.* 1982, Andersson *et al.* 1986, Mikaelsson *et al.* 1983). The reconstitution of FVIII activity from isolated heavy and light chains requires the presence of divalent cations, with Mn^{2+} having the strongest effect followed by Ca^{2+} and Co^{2+} (Fay 1988).

The incidence of haemophilia A is approximately 1 in 10,000 males, by the inheritance of an autosomal recessive pattern (Gitschier *et al.* 1984). Without treatment, haemophiliacs experience haemorrhage into joints and muscles, are easily bruised, and suffer prolonged and potentially fatal postoperative haemorrhages.

Restoration of plasma FVIII levels to greater than 1% of normal with plasma-derived commercial concentrates containing FVIII significantly reduces disease symptoms and markedly improves the quality of life for afflicted individuals (Aronson 1990, Esmon *et al.* 1990).

Although quite beneficial to most patients, treatment with some FVIII products can also have deleterious effects. In addition to the risk of viral transmission via plasma-derived FVIII products (Fletcher *et al.* 1983, Ragni *et al.* 1983), and the production of inhibitory antibodies, capable of neutralising FVIII (Shapiro and Hutlin 1975), treatment with cryoprecipitate or FVIII concentrates may result in functional and phenotypical immune deficiencies (Lederman *et al.* 1983, Menitove *et al.* 1983, Schulman 1991, Allersma et 1996). These immune deficiencies include impaired monocyte and phagocyte function as well as abnormalities of T cell subsets, irrespective of HIV infection (Moffat *et al.* 1985).

FVIII concentrates can be classified on the basis of specific activity (expressed in IU/mg total protein in the final product state) as either *intermediate* (1-5 IU/mg protein), *high* (10-200 IU/mg) or *very high* purity (1000-3000 IU/mg). *Intermediate* purity products are prepared by precipitation reactions and often contain high levels of fibrinogen and fibronectin as well as a number of other non-FVIII proteins. The yields from these processes are generally high. *High* purity products have reduced yields but significantly lower amounts of non-FVIII proteins, and are prepared using conventional chromatography techniques. *Very high* purity products contain essentially FVIII alone, and include monoclonal antibody purified concentrates and recombinant products. However, albumin is often added back into these products at the end of the manufacturing process to stabilise the product and to avoid Factor VIII dispersion on the glass wall of the container resulting in a lower specific activity than several high purity products. If the albumin content is subtracted from high or very high purity Factor VIII concentrates, figures in the range of 10-200 IU/mg or 1000-3000 IU/mg are obtained for specific activity, respectively.

Fractionation methods have been developed with the use of monoclonal antibodies directed against the FVIII molecule or von Willebrand Factor antigen (vWF:Ag) which successfully removed FVIII from cryoprecipitate during immunoaffinity chromatography. The protein content of these concentrates is dramatically decreased. Monoclonal antibody purified concentrates show a very high specific activity and are virtually free of extraneous plasma proteins. The achievement of a very low protein content seems to be strongly advisable because there is increasing evidence that immunodeficiency in multi-transfused haemophiliacs can be related not only to HIV infection but also the allogenic protein overload derived from replacement therapy (Morfini 1989).

The problem of virus transmission among the haemophilia population has been recognised since the early 1970s, hepatitis C virus and later HIV being the major viruses transmitted by clotting factor concentrates, and more recently hepatitis A virus and parvovirus B19. In order to reduce or abolish the virus load a variety of chemical and/or physical processes as well as methods of heat treatment under different experimental conditions (dry, steam, wet heat) have been introduced into the different stages of clotting factor concentrate production. The principal methods used are as follows:

- dry heat on freeze dried final product
- pasteurisation
- vapour heating under pressure
- solvent detergent treatment.

Terminal dry heat treatment is a popular methodology introduced in the late 1980s to ensure a high degree of viral safety (Winkelman *et al.* 1989). Coagulation factor concentrates that are heated at 80°C for 72 hours have an excellent safety record (Rizza *et al.* 1993, Cash 1988, Skidmore *et al.* 1990).

The use of sugars as stabilisers of biologically active proteins has been previously disclosed in US patents 2,826,533 (1958) and 4,089,944 (1978). Since the FVIII molecule is a heat sensitive protein, FVIII concentrates of intermediate purity have been stabilised with different concentrations of various sugars, lyophilised and exposed to dry heat regimes of 80°C for 72 hours (Roberts 1995).

The stabilising effect of a commercially available synthetic mixture of 14 synthetic amino acids (Synthamin 17) designed for intravenous use as a nutrient has also been extensively documented for the large scale manufacture of intermediate FVIII concentrates. Intermediate purity FVIII concentrate (1 IU/mg) was stabilised by the addition of Synthamin 17 when heated at 60°C for 48 hours (Benny *et al.* 1988). Synthamin 17 added in amounts of up to 4mg per unit FVIII, to a cryoprecipitate FVIII concentrate, resulted in better solubility and stability on storage. Heating the concentrate at 70°C for 16 hours with 1mg per unit maintained solubility and activity (Margolis and Eisen 1984).

The synthetic amino acid mixture was used for the large scale manufacture of an intermediate FVIII concentrate (1 IU/mg) using controlled pore glass adsorption chromatography. Heat stability of the freeze dried material was tested and no loss of FVIII activity was shown after 30 hours at 70°C compared with other intermediate purity concentrate preparations tested which exhibited approximately 25% of activity after only hours at 70°C and solubility was significantly impaired (Margolis *et al.* 1984, Austen 1979). Similar FVIII preparations have been stabilised by addition of the synthetic amino acid mixture (1.6% w/v) in the final product. The lyophilised material heated at 60°C for 72 hours reduced the FVIII loss to less than 10% compared with a non-amino acid stabilised concentrate, which resulted in the loss of 30% of FVIII:C activity (McGrath *et al.* 1985).

The clinical experience reported has been favourable. Patients infused with heat-treated material had a similar mean *ex vivo* recovery not significantly different from that observed with the non heated concentrates. The mean half life of both

heated and on heated material were reported to be identical. Stabilisation with Synthamin 17 and heat treatment does not influence the *in vivo* recovery and haemostasis was uniformly effective, with no adverse side effects being reported (Ockelford *et al.* 1987).

Established FVIII products are indeed true concentrates and unlike pure plasma products such as albumin or immunoglobulin, FVIII is only a small fraction of the protein contained in the concentrates (i.e. <1%), the majority being fibrinogen, immunoglobulin, fibronectin and many others. The presence of such proteins has also been shown to help stabilise the FVIII molecule over the terminal dry heat stage.

Concentrate purity is now a very important prerequisite to be taken into account when developing new FVIII concentrates. The current trend is towards increasing the purity of FVIII preparations, purer products will have less fibrinogen/fibronectin. However purity itself further complicates the product stability over the terminal dry heat step. The protein content of high purity concentrates is dramatically decreased. Monoclonal antibody purified concentrates show a very high specific activity and are virtually free of extraneous plasma proteins. However, as noted above albumin is often added at the end of the manufacturing process to stabilise the product. It is well known that albumin has a low risk for viral transmission and is possibly less antigenic than other proteins due to its low molecular weight. Whether albumin can be considered as an essential component of the protein overload and thereby of the immune depression observed in heavily-infused patients is still a matter of controversy. Cohn Fractionation Albumin solutions used to stabilise high purity FVIII concentrates are unlikely to be more than 95% pure and so trace amounts of other proteins may therefore be added back into the FVIII concentrates. However, this practice largely defeats the purpose of purification.

The first documented report of a high purity FVIII concentrate which was shown to be stable at ambient temperatures and at pasteurisation temperatures (60°C) in the absence of human serum albumin was recently disclosed in International Patent

Publication WO 96/22107. The addition of trehalose at 0.15 to 2.5mg per unit FVIII to a recombinant FVIII preparation showed excellent stability of FVIII activity when stored at 60°C for an extended period of time in the complete absence of human serum albumin. Trehalose is known to be a highly effective stabilising agent for delicate proteins as disclosed in US Patent No. 4,891,319 and Australian Patent No. 591160, enabling proteins to be dried at temperatures above freezing.

The stability of FVIII concentrates has been well established using sodium citrate as an anticoagulant in plasma fractionation. Early studies with fibrinogen preparations as disclosed in US Patent No. 2,826,533 illustrated the anticoagulant and solubilising properties of sodium citrate. Citrate was also reported to have a marked stabilising effect on early preparations of chromatographically purified FVIII concentrates (Hynes *et al.* 1969) which helped establish the use of 20mM trisodium citrate in commercial FVIII concentrates (Newman *et al.* 1971). FVIII activity shows a biphasic deterioration in the presence of citrate (Preston 1967). This loss of activity stems from the chelation by citrate groups of calcium ions which are essential for maintaining the normal structure of the FVIII molecule. Formulating during processing by adding calcium chloride to maintain physiological levels of ionised calcium stabilises FVIII activity (Fay 1988).

The present invention provides improved stabilising compositions which are particularly, but not exclusively, directed to the stabilisation of high and very high purity Factor VIII concentrates as discussed above. The present invention also provides stabilising compositions for these concentrates which avoid the necessity for adding back albumin, although albumin may still be added if desired and acceptable.

SUMMARY OF THE INVENTION

According to a first aspect of the present invention, there is provided a dried, heat-treated product comprising a heat labile, biologically or therapeutically active

protein or peptide preparation, and a stabilising effective amount of a composition comprising sucrose, trehalose and at least one amino acid.

In yet another aspect, the present invention provides a method of preparing a dried, heat-treated product comprising a heat labile, biologically or therapeutically active protein or peptide preparation, which comprises (i) adding a stabilising effective amount of a composition comprising sucrose, trehalose and at least one amino acid to said protein or peptide preparation; (ii) drying said admixture; and (iii) heat-treating said dried product.

In yet another aspect, the invention provides a composition for use in the stabilisation of a heat labile, biologically or therapeutically active protein or peptide preparation, comprising sucrose, trehalose and at least one amino acid.

The terms "drying" and "dried" are used herein in the broad sense to refer to removal of water from an aqueous product and products obtained thereby, and include in particular the freeze drying process (also known as lyophilisation).

The terms "heat-treating" and "heat-treated" are used herein to refer to methods of heat treatment in order to reduce or abolish a virus load in a biologically or therapeutically active product and products obtained thereby, and include dry heat treatment at greater than 75°C for up to 72 hours, more particularly dry heat treatment at 80°C for 72 hours.

In one particular embodiment, the present invention comprises a dried Factor VIII preparation comprising a Factor VIII concentrate, and a stabilising effective amount of a composition comprising sucrose, trehalose and at least one amino acid.

Preferably, the Factor VIII concentrate is a high purity (10 - 200 IU/mg) or very high purity (1000-3000 IU/mg) product.

References herein to a "high purity" or "very high purity" Factor VIII concentrate should be understood as referencing to the purity of the concentrate prior to the addition of a protein stabiliser.

Alternatively, the protein or peptide preparation may be a von Willebrand Factor concentrate.

Optionally, the stabilising composition in accordance with the present invention may also comprise albumin, particularly human serum albumin.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers."

DETAILED DESCRIPTION OF THE INVENTION

The formulations of the present invention have the potential to provide another alternative to the adding back of protein such as human serum albumin (HSA) as a stabilising agent for high purity FVIII concentrates. Whilst the use of albumin in the composition of this invention is optional, the addition of albumin is not an ideal option because it adds a complex component to the product formulation. The formulations detailed in this invention relate to defined chemical additives containing specific components designed to prevent adsorption, and to maintain the stability and solubility of high purity FVIII concentrates exposed to heat treatment regimes, in particular the dry heat treatment of lyophilised product heated at 80°C for 72 hours. The technology of the current invention offers many advantages over the practice of adding back albumin to stabilise high purity FVIII concentrates and the use of trehalose as disclosed in International Patent Publication WO 96/22107. The major advantages are highlighted below:

(i) Coagulation factor concentrates that are heated at 80°C for 72 hours have an excellent viral safety record. It has not been thought possible to prepare a high purity dried blood FVIII concentrate which is stable over this severe dry heat treatment step without the addition of albumin.

(ii) Trehalose as disclosed in WO 96/22107 may be used to stabilise a dried blood factor composition which undergoes heat treatment at pasteurisation temperatures (60°C). The amount of trehalose used in some of the formulations in the present invention when used alone is insufficient to produce a stabilising effect at temperatures greater than 60°C (i.e. 80°C for 72 hours). However, trehalose in combination with other chemical additives, sugars and amino acids as disclosed in this invention provides significant stability to high purity FVIII concentrates undergoing severe dry heat treatment.

(iii) The disclosed formulations provide a method of preparing aqueous solutions containing FVIII, which may be freeze dried at temperatures greater than 25°C.

(iv) The chemical compounds, sugars and amino acids used in the disclosed formulations can be chemically produced in large quantities under GMP conditions. By contrast, human serum albumin derived from plasma introduces considerable problems of purification since it is essential that the protein is free of viral contamination. Using recombinant HSA to overcome these problems is expensive but it is also derived from animals cells or bacterial culture, and therefore the risk of contamination by infectious agents or unknown materials is possible.

In the stabilising composition of the present invention, sucrose is preferably present in amounts of from 0.5 to 10% by weight, more preferably from 2.5 to 5% by weight. Similarly, trehalose is preferably present in amounts of from 0.5 to 5% by weight, more preferably from 1.25 to 2.5% by weight.

The amino acid which is incorporated into the stabilising composition is preferably lysine, and the amino acid is preferably incorporated in a molar amount of from 0.05 to 1M, more preferably 0.1 to 0.5M. Alternatively, however, the amino acid may be incorporated as a mixture of amino acids, particularly the mixture available commercially as Synthamin 17, or one or more of the amino acids present in Synthamin 17. Synthamin 17 is a formulation of amino acids as follows:

Synthamin 17

Essential Amino Acids (mg/100mL)	
L-Isoleucine	(600)
L-Leucine	(730)
L-Lysine	(580)
L-Methionine	(400)
L-Phenylalanine	(560)
L-Threonine	(420)
L-Tryptophan	(180)
L-Valine	(580)

Nonessential Amino Acids (mg/mL)	
L-Alanine	(2070)
L-Arginine	(1150)
L-Histidine	(480)
L-Proline	(680)
L-Serine	(500)
Glycine	(1030)

Such a mixture of amino acids is preferably used in the stabilising composition of the present invention in amounts of from 0.5 to 5% by weight, more preferably from 2 to 3% by weight.

As previously described, albumin (such as human serum albumin) may optionally be incorporated in the stabilising composition of the present invention. When incorporated, albumin may be present in an amount of from 0.1 to 10 mg/mL of the Factor VIII concentrate prior to freeze drying.

Whilst the present invention is principally directed towards the stabilisation of FVIII concentrates, particularly on drying high and very high purity FVIII concentrates, the compositions of the present invention may also be used in the stabilisation of other biologically or therapeutically active preparations, particularly those which are to be dried or lyophilised and subsequently treated to dry heat treatment as a viral inactivation step. Such other biologically or therapeutic preparations include, for

example, other blood, plasma or serum fractions, enzymes, antibodies, antigens, vaccine components and the like.

Further features of the present invention are more fully described in the following Example(s). It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

EXAMPLE

This Example relates to a stabilised, dried Factor VIII preparation comprising a high purity Factor VIII concentrate.

Method of Manufacture

Fresh frozen plasma (FFP) is thawed at temperatures below 5°C and the FVIII-rich cryoprecipitate is collected by centrifugation. The FVIII is extracted with Tris (hydroxymethyl) methylamine (Tris) buffer. Levels of unwanted proteins, principally fibrinogen, fibronectin, immunoglobulin and albumin, are reduced by precipitation with heparin followed by reprecipitation of FVIII with sodium chloride/glycine buffer. The purified FVIII is redissolved in a sodium chloride-Tris-citrate buffer containing sucrose and a low level of calcium chloride. The dissolved precipitate is filtered, treated with solvent/detergent and incubated. The mixture is then filtered and chromatographed on a Sephacryl S400 column pre-equilibrated in the same buffer. The FVIII-rich eluate (≥50 IU/mg total protein) is then concentrated by ultrafiltration against the same buffer and chemical stabilisers added to the retentate. The bulk formulated concentrate is sterile filtered, dispensed, freeze dried and heat treated at 80°C for 72 hours.

Freeze Drying/Dry Heat Treatment

The freeze drying cycle proceeds under conditions of programmed temperature/vacuum/timing for approximately 100 hours. The formulated product is loaded into a freeze dryer and the shelves cooled to -50°C. The vacuum is applied and the temperature ramped up to 50°C. The finished lyophilised product is then heated in a hot air oven at 80°C for 72 hours.

FVIII Procoagulant Activity

FVIII procoagulant activity was measured throughout the process using a chromogenic assay kit (Chromogenix, Sweden).

Samples from each batch were taken at various stages throughout the process (formulated bulk, freeze dried, and dry heated product) and tested for FVIII procoagulant activity. The results are shown in the following Table in comparison with an intermediate purity (4-5 IU/mg) product, AHF(HP), and the high purity concentrate formulated with human serum albumin (used as a stabiliser in a number of commercial high purity concentrates).

The FVIII concentrates used in the experiments detailed in this invention were formulated in a sodium chloride/Tris/citrate buffer containing sucrose and a low level of calcium chloride. The stability of FVIII concentrate using this buffering system has been well established. However, this formulation alone provided little protection of the FVIII molecule in the high purity product over the terminal dry heat treatment step. For the high purity product, a 33% loss of FVIII activity was reported, compared with the intermediate purity product in the same buffer where only 13% of the FVIII activity was lost over the same step, highlighting the protective effect of the high levels of protein contained in this concentrate. Similarly, the high purity product stabilised with added albumin only lost 21% FVIII activity. The post dry heated results also showed that additional sugars such as sorbitol, maltose and sucrose alone produced little or

no stabilisation over the dry heat treatment step. Significant losses of FVIII activity were observed from the formulated bulk concentrate to post terminal dry heat treatment. Similarly, the post drying results showed that it was not possible to dry heat the purity concentrate successfully when trehalose (45% loss of FVIII activity) or Synthamin 17 (25% loss of FVIII activity) were also alone.

Trehalose in combination with sucrose also produced significant losses in FVIII activity (61%). However, with a composition of trehalose in combination with sucrose and lysine, and with a composition of sucrose, trehalose, lysine in combination with other amino acids (Synthamin 17), it was possible to dry heat FVIII successfully in the absence of human serum albumin.

These formulations all produced results similar to the high purity concentrate stabilised with human serum albumin, and from the results disclosed it can be concluded that a high purity FVIII concentrate can indeed be successfully freeze dried and dry heated in a chemical based formulation in the absence of HSA.

Table 1 Percentage of FVIII activity loss from formulation to dry heat treatment with various stabilisers.

Formulation Composition	No. of Batches	% FVIII Activity Loss	
		Dry Heat	Formulation to Dry Heat
Buffer alone	5	33.2	44.1
Intermediate purity product AHF (HP)	4	12.6	14.8
Albumin (10 mg/mL)	7	20.9	26.6
5% sorbitol	1	98.3	98.8
5% sucrose	2	46.6	78.4
10% sucrose	1	39.9	44.8
2.5% maltose	1	89.3	91.3
2.5% trehalose	1	43.3	45.1
2.5% sucrose + 1.25% trehalose	1	60.6	63.3
2% Synthamin 17 + electrolytes	1	25.1	41.0
2.5% sucrose, 1.25% trehalose, 0.5M lysine	7	8.8	29.4
2.5% sucrose, 1.25% trehalose, 0.1M lysine, 2% Synthamin 17 + electrolytes	3	15.8	25.5

REFERENCES:

Allersma DP, Smid WM, van der Does JA, van der Meer J, Briet E. Effects of chronic factor VIII substitution on immune parameters in HIV seronegative haemophiliacs: a comparison between factor VIII cryoprecipitate and factor VIII concentrate. *Thromb. Haemostas.* (1996); **75**(2):261-6.

Andersson LO, Forsman N, Huang K, Lundin A, Pavlu B, Sadberg H, Sewerin K, Smart J. Isolation and characterisation of human factor VIII: molecular forms in commercial factor VIII concentrate , cryoprecipitate and plasma. *Proc.Natl. Acad. Sci.* (1986); **83**:2979-83.

Aronson D. The development of the technology and capacity for the production of factor VIII for the treatment of haemophilia A. *Transfusion* (1990); **30**:8:748-58.

Austen D. The chromatographic separation of factor VIII on aminohexyl Sepharose. *Br. J. Haematol.* (1979); **43**(4):669-74.

Benny A, Ockelford P, John A, Scott R, Woodfield D, Berry E. Influence of heat treatment on FVIII:C recovery from freeze dried cryoprecipitate. *J. Clin. Pathol.* (1988): **41**:945-7.

Cash J. Coagulation factor VIII concentrates and the market place. *Lancet* (1988); ii:814-6.

Eaton D, Rodrigez H, Vehar GA. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. Biochemistry (1986); **25**:505-12.

Eaton DL, Hass PE, Riddle L, Mather J, Wieve M, Gregory T, Vehan G. Characterisation of recombinant Human Factor VIII. *J. Biol. Chem.* (1987); **262**(7): 3285-90.

Esmon PC, Kuo HS, Fournel MA. Characterization of recombinant factor VIII and a recombinant factor VIII deletion mutant using a rabbit immunogenicity model system. *Blood* (1990); **76**(8):1593-1600.

Fass D, Knutson G, Katzmann J. Monoclonal antibodies to porcine factor VIII coagulant and their use in the isolation of active coagulant protein. *Blood* (1982); **59**(3):594-600.

Fay PJ, Anderson MT, Chavin SI, Marder VJ. The size of human VIII heterodimers and the effects produced by thrombin. *Biochem. Biophys. Acta* (1986); **871**:268-78.

Fay P. Reconstitution of human factor VIII from isolated subunits. *Arch. Biochem. Biophys.* (1988); **262**(2):525-31.

Fletcher ML, Trowell JM, Craske J, Pavier K, Rizza CR. Non-A and non-B hepatitis after transfusion of factor VIII in infrequently treated patients. *Br. Med. J.* (1983); **287**:1754-7.

Foster PA, Zimmerman TS. Factor VIII structure and function. *Blood Reviews* (1989); **3**:180-91.

Fulcher CA, Zimmerman TS. Characterisation of the human factor VIII procoagulant protein with a heterologous precipitating antibody. *Proc.Natl. Acad. Sci.* (1982); **798**:1648-52.

Fulcher CA, Roberts JF, Holland LZ, Zimmerman TS. Human factor VIII procoagulant protein: monoclonal antibodies define precursor-product relationships and functional epitopes. *J. Clin. Invest.* (1985); **76**:177-24.

Gitscher T, Wood WI, Goralka TM. Characterisation of the human factor VIII gene. *Nature* (1984); **312**:326-30.

Hynes H, Owen C, Bowie E. Thompson J. Citrate stabilisation of chromatographically purified factor 8. *Blood* (1969); **34**(5):601-9.

Lederman MM, Ratnoff OD, Scillian JJ, Jones PK, Schacter B. Impaired cell-mediated immunity in patients with classic hemophilia. *New Engl. J. Med.* (1983); **308**(2):79-83.

Levine PH. Clinical manifestations and therapy of hemophilias A and B. *Hemostat-Thromb.* RW Coleman, Philadelphia, Lippincott (1987):97-111.

Margolis J, Eisen M. Stabilising effect of amino acids on Factor VIII in lyophilised cryoprecipitate. *Lancet* (1984); Dec 8:1345.

Margolis J, Galiovich C, Rhoades P. A process for preparation of high purity factor VIII by controlled pore glass treatment. *Vox Sang.* (1984); **46**:341-3.

McGrath K. Thomas K, Herrington R, Turner P, Taylor L, Ekert H, Schiff P, Gust I. Use of heat treated clotting factor concentrates in patients with haemophilia and a high exposure to HTLV-111. *Med. J. Aust.* (1985); **1431**1-13.

Menitove JE, Aster RH, Casper JT, Lauer SJ, Gottschall JL, Williams JE, Gill JC, Wheeler DV, Piakoski V, Kirchner P, Montgomery RR. T-lymphocyte subpopulations in patients with classic haemophilia treated with cryoprecipitate and lyophilized concentrates. *new Engl. J. Med.* (1983); **308**(2):83-6.

Mikaelsson M, Forsman N, Oswaldsson U. Human factor VIII: a calcium-linked protein complex. *Blood* (1983); **62**(5):1006-15.

Moffat EH, Bloom AL, Jones J, Matthews N, Newcombe RG. A study of cell mediated and humoral immunity in haemophilia and related disorders. *Br. J. Haematol.* (1985); **61**:157-67.

Morfini M, Rafanelli D, Filimberti E, Cinotti S, Piazza E, Longo G, Rossi Ferrini P. Protein content and factor VIII complex in untreated, treated and monoclonal factor VIII concentrates. *Thromb. Res.* (1989; **56**:168-78.

Newman J, Johnson A, Karpatkin M, Puszkin S. Methods for the production of clinically effective intermediate and high purity factor VIII concentrates. *Br. J. Haematol.* (1971); **21**(1):1-20.

Ockelford P, Benny A, Berry E, Johns A, Woodfield D. Controlled pore glass factor VIII concentrate: influence of heat treatment. *Pathology* (1987); **19**(1):56-7.

Pittmann DD, Kaufman RJ. Indication of the cleavage site(s) requirements for activation and inactivation of factor VIII. *Blood* (1986); **68**:352a.

Preston A. The factor 8 activity in fresh and stored plasma. *Br. J. Haematol.* (1967); **13**(1):42-59.

Ragni MV, Lewis JH, Spero JA, Bontempo FA. Acquired-immunodeficiency-like syndrome in two haemophiliacs. *Lancet* (1983); January:213-4.

Rizza C, Fletcher M. Kernoff P. Confirmation of viral safety of dry heated factor VIII concentrate (8Y) prepared by Bio Products Laboratory. *Br. H. Haematol.* (1993); **84**(2):269-72.

Roberts P. Severe heat treatment of freeze-dried coagulation factor concentrates. *Vox Sang.* (1995); **68**(1):61-2.

Rotblat F, O'Brien D, O'Brien F, Goodall A, Tuddenham E. Purification of human factor VIII:C and its characterisation by Western blotting using monoclonal antibodies. *Biochemistry* (1985); **24**(16):4294-4300.

Schulman S. Effects on factor VIII concentrates on the immune system in hemophilic patients. *Ann. Hematol.* (1991); **63**:145-51.

Shapiro SS, Hutlin M. Acquired inhibitors to the blood coagulation factors. *Semin. Thromb. Haemost.* (1975); **1**:336-41.

Skidmore S, Pasi K, Mawson S, Williams M, Hill F. Serological evidence that dry heating of clotting factor concentrates prevents transmission of nonA nonB hepatitis. *J. Med. Virol.* (1990); **30**(1):50-2.

Toole J, Knopf J, Wozney J, Sultzman L, Buecker J, Pittman D, Kaufman R, Brown E, Shoemaker C. *et al.* Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature* (1984); **312**(5992):342-7.

Walker F, Chavin S, Fay P. Inactivation of factor VIII by activated protein C and protein S. *Arch. Biochem. Biophys.* (1987); **252**(1):322-8.

Weiss HJ, Sussman II. Stabilisation of factor VIII in plasma by the von Willebrand Factor. J Clin Invest (1977); **60**:390-404.

Winkelman L, Owen NE, Evans DR, Evans H, Haddon ME, Smith JK, Prince PJ, Williams JD, Lane RS. Severely heated therapeutic factor VIII concentrate of high specific activity. *Vox Sang.* (1989); **57**:97-103.

CLAIMS:

1. A dried, heat-treated product comprising (i) a heat labile, biologically or therapeutically active protein or peptide preparation, and (ii) a stabilising effective amount of a composition comprising sucrose, trehalose and at least one amino acid.

2. A product as claimed in claim 1, wherein said product has been dried by freeze drying.

3. A product as claimed in claim 1, wherein said product has been dry heat-treated at greater than 75°C for up to 72 hours.

4. A product as claimed in claim 1, wherein said at least one amino acid is lysine.

5. A product as claimed in claim 1, wherein said at least one amino acid comprises a mixture of amino acids as follows:

Essential Amino Acids (mg/100mL)	
L-Isoleucine	(600)
L-Leucine	(730)
L-Lysine	(580)
L-Methionine	(400)
L-Phenylalanine	(560)
L-Threonine	(420)
L-Tryptophan	(180)
L-Valine	(580)

Nonessential Amino Acids (mg/mL)	
L-Alanine	(2070)
L-Arginine	(1150)
L-Histidine	(480)
L-Proline	(680)
L-Serine	(500)
Glycine	(1030)

6. A product as claimed in claim 1, wherein said protein or peptide preparation is a von Willebrand Factor concentrate.

7. A product as claimed in claim 1, wherein said protein or peptide preparation is a Factor VIII concentrate.

8. A product as claimed in claim 7, wherein said Factor VIII concentrate is a high purity concentrate.

9. A product as claimed in claim 7, wherein said Factor VIII concentrate is a very high purity concentrate.

10. A product as claimed in any one of claims 1 to 9, wherein said composition further comprises albumin.

11. A product as claimed in claim 10, wherein said albumin is human serum albumin.

12. A method of preparing a dried, heat-treated product comprising a heat labile, biologically or therapeutically active protein or peptide preparation, which comprises (i) adding a stabilising effective amount of a composition comprising sucrose, trehalose and at least one amino acid to said protein or peptide preparation; (ii) drying said admixture; and (iii) heat-treating said dried product.

13. A method as claimed in claim 12, wherein said drying step (ii) comprises freeze-drying.

14. A method as claimed in claim 12, wherein said heat-treating step (iii) comprises dry heat treatment at greater than 75°C for up to 72 hours.

15. A method as claimed in claim 12, wherein said at least one amino acid is lysine.

16. A method as claimed in claim 12, wherein said at least one amino acid comprises a mixture of amino acids as follows:

Essential Amino Acids (mg/100mL)		Nonessential Amino Acids (mg/mL)	
L-Isoleucine	(600)	L-Alanine	(2070)
L-Leucine	(730)	L-Arginine	(1150)
L-Lysine	(580)	L-Histidine	(480)
L-Methionine	(400)	L-Proline	(680)
L-Phenylalanine	(560)	L-Serine	(500)
L-Threonine	(420)	Glycine	(1030)
L-Tryptophan	(180)		
L-Valine	(580)		

17. A method as claimed in claim 12, wherein said protein or peptide preparation is a von Willebrand Factor concentrate.

18. A method as claimed in claim 12, wherein said protein or peptide preparation is a Factor VIII concentrate.

19. A method as claimed in claim 18, wherein said Factor VIII concentrate is a high purity concentrate.

20. A method as claimed in claim 18, wherein said Factor VIII concentrate is a very high purity concentrate.

21. A method as claimed in any one of claims 12 to 20, wherein said composition further comprises albumin.

22. A method as claimed in claim 21, wherein said albumin is human serum albumin.

23. A composition for use in the stabilisation of a heat labile, biologically or therapeutically active protein or peptide preparation, comprising sucrose, trehalose and at least one amino acid.

24. A composition as claimed in claim 23, wherein said at least one amino acid is lysine.

25. A composition as claimed in claim 23, wherein said at least one amino acid comprises a mixture of amino acids as follows:

Essential Amino Acids (mg/100mL)		**Nonessential Amino Acids (mg/mL)**	
L-Isoleucine	(600)	L-Alanine	(2070)
L-Leucine	(730)	L-Arginine	(1150)
L-Lysine	(580)	L-Histidine	(480)
L-Methionine	(400)	L-Proline	(680)
L-Phenylalanine	(560)	L-Serine	(500)
L-Threonine	(420)	Glycine	(1030)
L-Tryptophan	(180)		
L-Valine	(580)		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00682

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 47/18, 47/26, 38/37, A61L 2/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K} and Key Word Search Terms Indicated
A61L} Below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT (WPAT) }
CA (STN) } Sucrose, Trehalose, Amino Acid, Lysine, Factor VIII, Protein, Heat
MEDLINE (STN) }

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 599543 A (TANABLE SEIYAKU CO., LTD.) 1 June 1994 Page 2 lines 26-30, Page 3 lines 21-23 and 32-34	1, 2, 4, 12, 13, 15, 23, 24
X	Patent Abstracts of Japan JP 8-187095 A (TOYOBO CO. LTD.) Abstract only	1, 2, 4, 10-15, 21-24
X	Derwent Abstract Accession No. 94-022829/03, Class B04 JP 05331071 A (ASAHI CHEM IND CO LTD) 14 December 1993	1, 2, 4, 12, 13, 15, 23, 24

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1998	29 OCT 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **J.G. HANSON** Telephone No.: (02) 6283 2262

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00682

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4297344 A (BEHRINGWERKE AKTIENGESELLSCHAFT) 27 October 1981	
A	US 4562072 A (BEHRINGWERKE AKTIENGESELLSHAFT) 31 December 1985	
A	EP 077870 A (THE GREEN CROSS CORPORATION) 4 May 1983	
A	Patent Abstracts of Japan JP 6-321805 A (ASAHI CHEM IND CO LTD) 22 November 1994	
A	US 4623717 A (MILES LABORATORIES, INC.) 18 November 1986	
A	WO 96/22107 A (QUADRANT HOLDINGS CAMBRIDGE LTD.) 25 July 1996	
A	WO 87/00196 A (QUADRANT BIORESOURCES LTD) 15 January 1987	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 98/00682

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	599543	JP	6157294	US	5650172		
US	4297344	DE	2916711	DK	1762/80	EP	18561
		ES	490682	ES	8100884	IL	59924
		JP	55145615				
US	4562072	CA	1208131	DE	3237512	EP	106269
		ES	526330	ES	8405620	IL	69927
		JP	59088427	PT	77464	ZA	8307506
EP	077870	ES	509941	ES	8302772	JP	58074617
		US	4446134				
US	4623717	CA	1187410	DK	986/81	EP	35204
		ES	500121	ES	8201827	JP	56139422
		US	4440679				
WO	96/22107	AU	44540/96	CA	2210872	EP	871476
WO	97/00196	EP	832031	NO	975884		

END OF ANNEX